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NOTE

***Chlamydia trachomatis* and *Chlamydophila abortus* induce the expression of secretory leukocyte protease inhibitor in cells of the human female reproductive tract**

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ABSTRACT

C. trachomatis and *C. abortus* are related Gram-negative intracellular bacteria that cause reproductive failure due to infertility (*C. trachomatis*) or abortion (*C. abortus*). These organisms target epithelial cells in the reproductive tract and/or placenta, but the innate immune mechanisms that lead to protection or pathology and disease are poorly understood. SLPI is an innate immune molecule which protects mucosal surfaces from infection and injury. *C. trachomatis* and *C. abortus* were found to induce SLPI mRNA and peptide expression in HeLa (cervical epithelium) and JEG-3 cells (trophoblast) respectively. Both cell lines constitutively expressed SLPI and, although infection enhanced this expression, killed organisms did not. These data demonstrate that *Chlamydia/Chlamydophila* grow in cells that express SLPI, suggesting that SLPI does not exert antimicrobial effects against these organisms. However, SLPI has multiple functions, and we speculate that it may play a role in controlling tissue inflammation and pathology.

Key words *Chlamydia*, innate immunity, secretory leukocyte protease inhibitor

C. trachomatis and *C. abortus*, both obligate intracellular bacterial pathogens belonging to the genus *Chlamydiae*, are associated with reproductive failure (1). *C. trachomatis* infects epithelial cells of the lower and upper reproductive tracts and has a strong association with infertility and ectopic pregnancy. These outcomes have been linked to inflammatory host immune responses (2). *C. abortus* infects placental trophoblast and causes abortion in sheep and humans. Again, this response is linked to local immunopathology (3). Both pathogens can induce chronic infections. The early events following primary (acute) infection are likely to be very important in determining

progression to chronic infection. Given that acute chlamydial infections are characterized by intense inflammation and mucosal infiltration of immune cells including neutrophils (4), and that *Chlamydiae* are susceptible to natural antimicrobials (5, 6), it was hypothesized that innate immunomodulatory molecules may play a role in chlamydial infection of epithelial cells in the reproductive tract.

SLPI is a highly conserved proteinase inhibitor that is constitutively expressed by epithelial cells, mast cells, neutrophils and macrophages. SLPI is thought to protect mucosal surfaces from damage during inflammation (7) acting as a neutrophil elastase inhibitor (8) and controlling

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List of Abbreviations: ANOVA, analysis of variance; *C. abortus*, *Chlamydophila abortus*; *C. trachomatis*, *Chlamydia trachomatis*; EB, elementary bodies; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IMDM, Iscove's modified Dulbecco's medium; LPS, lipopolysaccharide; mJ, millijoule; MOI, multiplicity of infection; *M. tuberculosis*, *Mycobacterium tuberculosis*; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; SLPI, secretory leukocyte protease inhibitor; TNF- α , tumor necrosis factor-alpha; UV, ultraviolet

inflammation by down-regulating TNF- α production in LPS-activated macrophages (9). SLPI also exhibits antimicrobial properties against both Gram-negative and Gram-positive bacteria (7).

Expression of SLPI at mucosal sites such as the respiratory and gastrointestinal tracts can be modulated, both positively and negatively, by pathogens. For example, *Staphylococcus aureus* (10) and *Helicobacter pylori* (11) can inhibit SLPI expression whereas HIV-1 (12) and *M. tuberculosis* (13) can augment SLPI expression. SLPI is expressed throughout the female reproductive tract (14) and in human trophoblasts (15). It is an antibacterial constituent of vaginal fluid (16) and cervical mucus (17). Furthermore, the bactericidal activity of uterine cell washes in culture can, at least partially, be inhibited by antibodies that neutralize SLPI (18). Thus, it is of interest to investigate modulation of SLPI expression by pathogens of the reproductive tract with a view to understanding the complex host-pathogen interactions that can lead to protection or immunopathology. To address our hypothesis, SLPI expression in HeLa 229 (derived from human cervical epithelium) and JEG-3 cells (derived from human placental chorionic epithelium) infected with *C. trachomatis* and *C. abortus* respectively, was investigated.

HeLa cells were grown to sub-confluence in IMDM, (Invitrogen, Paisley, UK) supplemented with 2% FBS (PAA laboratories, Teddington, UK) in 24 well plates (Corning Costar, High Wycombe, UK). Cells (2×10^5 cells in 1ml) were infected with *C. trachomatis* (Serovar E) at an estimated MOI of 0.1. The MOI was derived by evaluating the number of inclusion-forming units of the parent stock of *C. trachomatis* EB titrated on HEp-2 cells. Infected cells were fixed 48 hr after infection and inclusions stained using the 13/4 anti-chlamydial LPS monoclonal antibody followed by a rabbit anti-mouse FITC conjugated antibody. Uninfected cells served as controls. The cells were cultured for 4, 8, 24 or 48 hr, after which time the supernates were harvested and stored at -20°C until further analysis for the presence of SLPI peptide by ELISA (Hycult Biotech, Uden, the Netherlands). At harvest, the cells were washed with cold PBS and RNA isolated using the Qiagen RN easy mini kit (Qiagen, Crawley, UK) for analysis of the presence of mRNA encoding SLPI by quantitative real-time RT-PCR. All reagents and equipment used for cDNA synthesis and real-time RT-PCR analysis were from Applied Biosystems (Applied Biosystems, Warrington, UK) unless otherwise stated. One μg of total RNA was reverse transcribed using the Taqman Reverse transcription kit. For the real-time PCR, 50 ng of cDNA was amplified using the forward primer 5'-TGACACTGTGGCATCAAATG-3', the reverse primer 5'-CCCAGGCTTCCTCCTTGTT-3' and the probe 5'-GGATCCTGTTGACACCCCAAACC-3' using the Taqman Master Mix, on an ABI 7000 Prism real-

time thermo cycler. 18S rRNA was amplified in a duplex reaction using a validated primer: probe set as internal control and to allow quantitation relative to baseline uninfected control cells. Experiments were repeated at least three times. Due to evidence of increasing variability with increasing means, results were \log_{10} transformed prior to statistical analysis by two-way ANOVA which was used to generate 95% confidence intervals.

Relative to uninfected controls, SLPI mRNA expression was down regulated 4 hr after *C. trachomatis* infection ($P < 0.05$; Fig. 1a). However, 24 and 48 hr after infection there was a greater than 2.5 fold increase relative to uninfected cells (Fig. 1a). The increase in mRNA expression following infection was reflected in an increase in SLPI peptide in the culture supernates after 48 hr ($P < 0.05$; Fig. 1b). Measurement of SLPI peptide in the culture supernates revealed constitutive expression in uninfected cells. Despite the constitutive and induced expression, *C. trachomatis* grows well in HeLa cells suggesting that SLPI does not exert strong anti-chlamydial effects (19).

Since HeLa is a cell line widely-used to study *C. trachomatis* biology, the question of whether the phenomenon of SLPI induction is unique to that particular host-pathogen interaction is of interest. The closely-related organism *C. abortus* was therefore studied in a different natural target cell (trophoblast). Experiments were conducted using the same protocols as described for *C. trachomatis* infection of HeLa cells with minor modifications as outlined below. Briefly, JEG-3 chorionic trophoblast cells were grown to sub-confluence in 24 well plates and infected with *C. abortus* at an estimated MOI of 0.1, 1 or 10. The *C. abortus* stock (S26/3) was grown in HEp2 cells and titrated as described above for *C. trachomatis* with the exception that cells were fixed 72 hr after infection. A lysate of uninfected HEp2 cells and UV-killed *C. abortus* EB were used as further controls. The uninfected cell lysate was used at dilutions that matched those of the infected cells. EB were exposed to UV at 254 nm, 20 mJ and inactivation was confirmed by the failure of *C. abortus* to grow in HEp-2 cells as determined using the same staining protocol as that used for the titration. Supernates and cell lysates were harvested 24, 48 and 72 hr after infection and analyzed for SLPI protein and mRNA as described above. The results are shown in Figure 2.

A dose-dependent and time-dependent increase in SLPI mRNA expression was observed at MOI of 1 and 10 at 48 hr (2 and 7-fold respectively; $P < 0.05$ compared to uninfected cells) and 72 hr (4 and 8-fold respectively; $P < 0.05$ compared to uninfected cells, Fig. 2a). As for HeLa cells, JEG-3 trophoblasts were found to constitutively release SLPI peptide into the culture supernates (Fig. 2b). Again, a significant increase was observed following infection with

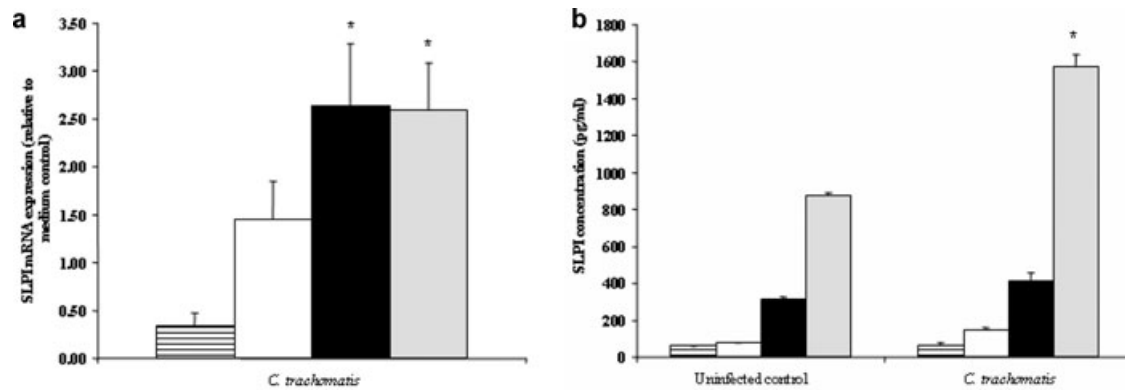


Fig. 1. SLPI expression in HeLa cells infected with *C. trachomatis* serovar E (MOI 0.1). (a) SLPI mRNA expression. Each value represents the fold change compared to the uninfected/untreated control cells at that time point. 4 hr (lined bar), 8 hr (white bar), 24 hr (black bar), 48 hr (grey bar). (b) SLPI protein secretion into the culture supernatant. 4 hour (lined bar),

8 hour (white bar), 24 hour (black bar), 48 hour (grey bar). Error bars represent the standard error of the means of four independent experiments. * $P < 0.05$ increase relative to medium alone values at the specific time point.

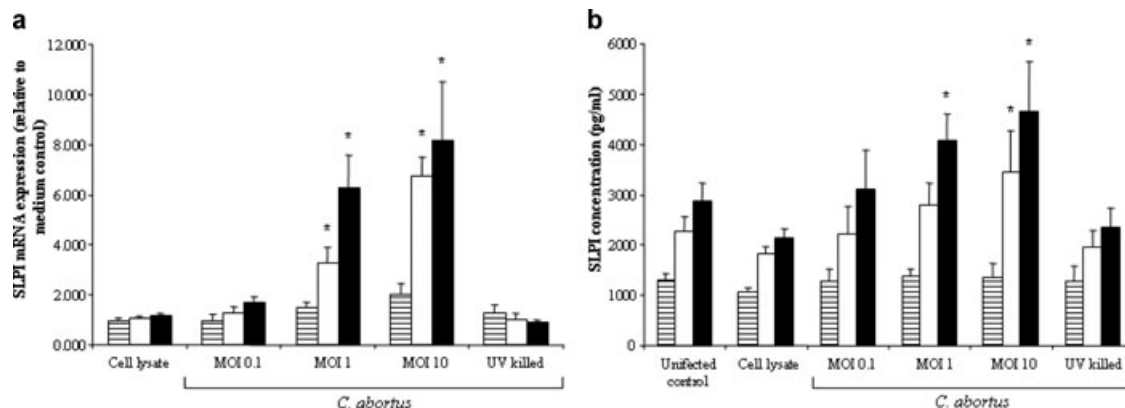


Fig. 2. SLPI expression in JEG-3 cells, infected with *C. abortus* at MOI 0.1, 1, 10, UV treated organisms or exposed to a HEp2 cell lysate prepared using the same method used for the propagation of *C. abortus*. (a) SLPI mRNA expression. Each value represents the fold change compared to the control uninfected sample (medium alone) at that time point.

(b) SLPI protein secretion into the culture supernatant. 24 hr (lined bar), 48 hr (white bar), 72 hr (black bar). Experiments were carried out on at least three separate occasions. Error bars represent the standard error of the means. * $P < 0.05$ different from medium control values at the specific time point.

C. abortus at MOI of 1 ($P < 0.05$) and MOI of 10 at 72 hr ($P < 0.05$) compared to uninfected controls (Fig. 2b).

However, there was no increase in SLPI mRNA expression or peptide when JEG-3 cells were exposed to UV-C-irradiated (killed) *C. abortus* or treated with cell lysates (Fig. 2a, b). The failure of killed organisms to induce SLPI expression by trophoblast indicates that chlamydial invasion and/or multiplication is an essential element in this process. It also suggests that the sensory mechanism that detects the organism, and subsequently promotes SLPI expression, is likely to be an intracellular receptor as opposed to a cell-surface exposed pattern recognition receptor.

These data demonstrate that *C. trachomatis* and *C. abortus* infection stimulate SLPI expression by cells in

the female reproductive tract. The requirement for invasion and/or multiplication is interesting since both live and heat-killed *M. tuberculosis* stimulate the expression of SLPI mRNA in macrophages (13). The production of antimicrobial molecules following chlamydial infection is not without precedent. Defensins have been found in patients with *C. trachomatis*-induced urethritis (20). However, given that both *C. trachomatis* and *C. abortus* grow very well in cells that constitutively express SLPI, and moreover induce further SLPI expression, we deduce that SLPI does not exert potent antimicrobial effects on these organisms. This raises the question as to the function of SLPI during chlamydial infection. We speculate that its role is to control inflammation in a manner that

supports chlamydial survival *in vivo*, possibly via mechanisms that interfere with neutrophil clearance of infection. This remains to be addressed.

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